Phenylketonuria—The Guthrie Screening Test

A Method of Quantitation, Observations on Reliability and Suggestions for Improvement

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■ *The Guthrie bacterial inhibition assay method of screening neonatal infants for phenylketonuria (PKU) initiated mass screening for inborn errors of metabolism. It is a simple, cheap procedure admirably suited to private local use as against private or public central use. However, using parallel fluorometric determinations as a basis for comparison, and 4 mg per 100 ml serum phenylalanine as a presumptive positive threshhold, the Guthrie test yielded 53 per cent "false negatives." Extrapolating from a combination of our data and reported phenylalanine levels at three days of age or less in proved PKU patients, it is estimated the Guthrie test might fail to detect one of 25 PKU patients screened at three days of age or less. Means to diminish this risk are considered.*

MASS SCREENING for the inborn metabolic error phenylketonuria, justified because the serious consequences of the condition can be greatly diminished upon early detection, was initiated by Guthrie,[3-5] who used a bacterial inhibition assay test. The subsequent development of a more specific, quantitative fluorometric method[2,10] for confirmation of screening results, and for proper control of phenylketonuric patients under treatment, was followed by adaptation of it to automated apparatus for use as a screening test.[7] Anyone who will take the trouble to perform screening studies in parallel by two methods, the GBIA (Guthrie Bacterial Inhibition Assay test) and MRF-AP (McCaman, Robins Fluorometric test, Automated Paper) will quickly confirm the substantial superiority of the latter. Why not, then, with recognition and tribute to its pioneering importance, shelve the GBIA method for good? We offer these considerations in trying to reach a decision:

- Since approximately 10,000 persons must be screened to detect one with PKU, the method employed should be the most reliable and the cheapest.
- A screening method may be quantitatively crude; as long as it always detects significant elevations of phenylalanine, it is reliable.
- Low cost per test can be achieved through volume testing. We believe it is of far-reaching importance that testing be done privately (and, in passing, we salute our Public Health Department for having the same belief). We believe also that

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local testing is preferable to centralized testing, public or private, provided it can be done as reliably and as cheaply.

- Provided it is reliable, the GBIA screening test, simple to do and read, is foremost as the method for small volume, local, private performance.

The arguments lead to a single critical question: Is the GBIA test reliable? Employed properly, can the GBIA test be depended on always to detect blood phenylalanine concentrations at that level we consider critical, whether it be 4 mg or 6 mg per 100 ml, or whatever we choose? Considering the minute fraction of positives and the crucial importance of initiating treatment as early as possible, the GBIA test must be 100 per cent reliable.

There has been some question raised about the reliability of the GBIA test as a method for screening for PKU in infants at an early age.[9,12] Irwin[9] reported that of 30 patients with serum phenylalanine levels of 4.1 mg per 100 ml and higher, only 11 had elevated values by the Guthrie method. In his series there were five patients with levels over 6.5 mg, and in four of these the Guthrie test also showed elevation. A recent extensive discussion of this matter by Partington and Sinnott[11] included brief consideration of "false negative" results. In their extensive studies, in which they quantitated zones of growth by measuring the diameter of the bacterial colonies they encountered no such instances.

"False positives" could contribute a serious problem if they were frequent but the incidence is not sufficiently great to make this important. We are concerned with the possible occurrence of "false negatives." Undocumented remarks at a PKU conference[1] in 1965 planted a seed of doubt. We realized we were totally dependent on the reliability of standards commercially available, that at times they appeared to behave differently in successive runs, that two different standards (for example, 4 mg and 6 mg) sometimes looked alike—in short, that we had no quality control.

Consequently we began a series of studies to determine the reliability of the GBIA method, and, if there was room for improvement, to bring it about in such a manner and with such devices as would neither appreciably diminish its simplicity nor increase its cost. Such efforts are continuing; this is an interim report based on results accruing from modifying the medium, quantitating the growth and utilizing data derived from studies not primarily intended for this purpose.

Materials and Methods

Densitometer

We realized we could not critically evaluate performance of the several standards in a given run, compare performance of successive runs or assess the usefulness of modifications of the medium unless we had some way of measuring bacterial growth. Scheel and Berry[12] and Partington and Sinnott[11] quantitated Guthrie tests by measuring the diameter of the zone of growth in millimeters. Close inspection of plates led us to conclude that whereas at times the margins of colonies appeared sharp, there were other times when they were indistinct; hence such measurements could be misleading.

To achieve quantitation at the lowest possible cost, we constructed a densitometer utilizing two selenium photocells (International Rectifier, No. B2M) in a series-aiding circuit. The reference and the reading cells are activated by readily available microscope illumination lamps (GE No. 1493). Utilizing a microammeter with zero at midscale (Triplett Model 320M) the circuits are balanced at zero by positioning the reference photocell in relation to its lamp with a screw drive. The circuit is next switched to a second microammeter with zero at the left end of the scale (Triplett Model 327PL). Polarity connections of the photocells are made so that anything placed in the path of the reading cell causes a deflection to the right, and its density is read in microamperes. To insure full use of the scale (and deflection can be altered by varying lamp intensity) a neutral density filter (Wratten 0.50 density, 32 per cent transmittance) determined by trial to be equal to the effects of bacterial growth around a disk soaked with blood containing 40 mg of phenylalanine per 100 ml is inserted into the reading cell path. Then the current supply is adjusted to give a reading of 40 microamperes.

Means to obtain a reasonably constant lamp current supply is the costliest part of the device. We have tried two combinations: A combination of 30-watt voltage stabilizer (Raytheon RVA-30S), a variable transformer (GE Variable Transformer 9H30AA10X) and a stepdown transformer (Chicago Standards Filament Transformer P-6456) to yield 6.3 volts alternating current; and a nickel cadmium battery pack (Edmund Scientific Co., No. 70,777) with attached charger to supply 7.2 volts direct current, regulated by a variable resistor (International Resistor Co., 2 ohm,

50 watt). With cost a primary consideration, we prefer the latter.

With this apparatus we measured the optical density of bacterial growth around various standards. The deflections recorded are a product of medium, disk and bacterial growth. As the blood-soaked disks are opaque at the light intensity used, they produce a constant effect. The medium and the bacterial growth are the two variables. Readings on unaltered media show variations from zone to zone, usually minor but definite. Were it possible to read the medium all around a disk, plus the growth, a growth density value closest to actuality would be obtained. This has not been possible to now but it is being achieved in a new form of culture container and densitometer under development. In our initial studies we determined the lowest medium density reading and used it as a base line for each plate.

Modified Medium

After experimenting with several substances as a medium, we employed 2,3,5 triphenyl tetrazolium, using 0.05 ml of a 1 per cent aqueous solution added to 50 ml of melted medium (Difco) at a temperature of approximately 55°C. This slowed rate of growth temporarily with interesting effects if plates were read after 20 hours instead of 16 hours of incubation as is done with the regular medium.

Results

Comparative Behavior of Standards on Regular and Tetrazolium Media

The results are given in Table 1 and are presented as graphs in Chart 1. On regular media, readings at 20 hours as compared with 16 showed less variation, but the spread between lowest and highest standards was no greater. On tetrazolium medium, read at 20 hours, the spread was considerably greater, not at the four lower concentrations (2, 4, 6 and 8 mg per 100 ml) but because of results with 12 and 20 mg per 100 ml standards, probably due to the red of the reduced tetrazolium-formazan. Variability was reduced, again only at the 12 and 20 mg per 100 ml concentration standards. The data as plotted can serve as a quality control chart.

The reddish-pink growth on tetrazolium as compared with the grey growth on regular media is possibly slightly easier to evaluate by visual inspection, but the difference is not great. Persons interested should determine for themselves whether or not it justifies one additional step in preparing the medium and reading at 20 instead of 16 hours.

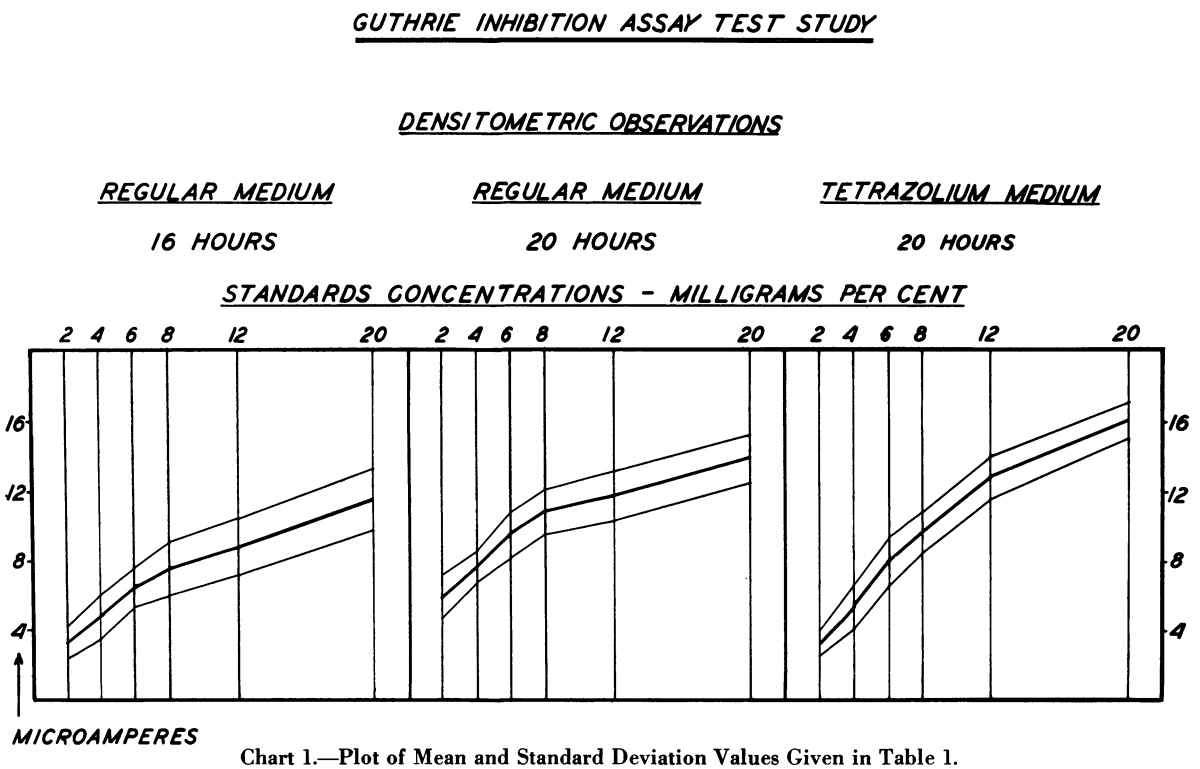

Chart 1.—Plot of Mean and Standard Deviation Values Given in Table 1.

TABLE 1.—*Densitometric Studies of Bacterial Growth Measured in Microamperes*

Standard (Per Cent)	Values	Regular Medium 16 Hours	Regular Medium 20 Hours	Tetrazolium Medium 20 Hours
2 mg	Mean	3.35	5.75	3.17
	S.D.*	0.91	1.24	0.73
	C.V.†	27.1	21.6	22.0
4 mg	Mean	4.83	7.62	5.34
	S.D.	1.31	0.96	1.31
	C.V.	27.1	12.6	24.5
6 mg	Mean	6.46	9.50	7.81
	S.D.	1.13	1.31	1.46
	C.V.	17.5	13.8	18.7
8 mg	Mean	7.53	10.78	9.60
	S.D.	1.54	1.30	1.17
	C.V.	20.1	12.1	12.2
12 mg	Mean	8.71	11.75	12.76
	S.D.	1.59	1.49	1.26
	C.V.	18.3	12.7	9.9
20 mg	Mean	11.41	13.79	16.05
	S.D.	1.73	1.39	1.06
	C.V.	15.2	10.1	6.6

*S.D.—Standard deviation.
†C.V.—Coefficient of variation.

Standards Discrepancies

The data obtained by densitometric observations on regular medium with 2, 4, 6, 8, 12 and 20 mg per 100 ml concentration standards, read at 16 and again at 20 hours, and on 50 tetrazolium plates with similar standards read at 20 hours, gave evidence of discrepancies in the behavior of the standards. As used here, the term *standards discrepancies* means similar densitometric readings for two (or more) standards, purportedly containing blood with different concentrations of phenylalanine. It is here emphasized that visual and densitometric observations often but not always coincide. Different "looking" standards may yield similar readings and similar "looking" standards may yield different densitometric readings, but such instances are exceptions and not the rule. We are concerned here with densitometric evaluations only. Table 2 lists standards discrepancies observed on regular and tetrazolium medium. They occurred in 16 of 50 sets of standards on regular medium and in only five of 50 sets of standards on tetrazolium medium; but the difference was due largely to discrepancies between 8 and 12 and 12 and 20 mg standards. At lower concentrations the kind of medium made no difference as to the incidence of such discrepancies.

TABLE 2.—*Standards Discrepancies*

Similar readings Standards, mg per 100 ml	Regular Medium Read at 16 Hours	Regular Medium Read at 20 Hours	Tetrazolium Read at 20 Hours
2 and 4	....	....	3
4 and 6	3	1	....
6 and 8	2	3	2
6 and 12	....	1	....
6, 8, 12	2	....	....
8 and 12	7	7	....
12 and 20	2	4	....
Totals	16	16	5

"False Negatives" and "False Positives"

Proceeding on the assumption that MRF-AP test values are true, and taking the arbitrary stand that a GBIA test reading of 4 mg per 100 ml or more is a presumptive positive, the term "false positive" denotes a GBIA value of 4 mg or more per 100 ml with an MRF value less than 4 mg, and the term

TABLE 3.—*Comparison of Blood Phenylalanine Determinations*

Fluorometric* Method Mg per 100 ml	Number	Guthrie† Method Mg per 100 ml	Number	Two Methods Agreed (Per Cent)	FP (Per Cent)	FN (Per Cent)
Less than 4	385	Less than 4	383	99.5	....	.....
		4 or more	2	.....	0.5	.....
4 or more	51	Less than 4	27	.....	....	53
		4 or more	24	47	....	.....
4.0–4.9	14	Less than 4	14	.....	....	100
		4 or more	0	.....	....	.....
5.0–5.9	19	Less than 4	8	.....	....	42
		4 or more	11	58	....	.....
6.0–6.9	10	Less than 4	5	.....	....	50
		4 or more	5	50	....	.....
7.0 or more	8	Less than 4	0	.....	....	.....
		4 or more	8	100	....	.....

*Fluorometric: Manual or automated McCaman-Robins method.
†Guthrie: Guthrie bacterial inhibition assay test.
FP: False positive; FN: False negative.

false negative denotes a GBIA value of less than 4 mg per 100 ml with an MRF value of 4 mg or more. A perfect screening test should produce neither "false positive" nor "false negative" results, but of the two kinds of error it is far more important to eliminate "false negatives."

Data summarized in Table 3 were derived from studies of 236 specimens from mature infants and 200 from premature infants (prematures are a valuable source of relatively high blood phenylalanine specimens). Each of the total of 436 specimens was examined by the GBIA method and by one or more MRF methods.

There were 385 specimens with less than 4 mg of phenylalanine per 100 ml by MRF testing and 383 had GBIA values less than 4 mg per 100 ml, an agreement of 99.5 per cent. Only two had GBIA values of 4 mg per 100 ml, a 0.5 per cent incidence of "false positives."

Fifty-one specimens had MRF values of 4 mg or more per 100 ml. Twenty-four of the GBIA values were in agreement, but for 27 the values were less than 4 mg per 100 ml, a 53 per cent incidence of "false negatives." In Table 3, data for various phenylalanine levels 4 mg per 100 ml and upward by 1 mg increments are listed. For all specimens with 7 mg or more by MRF values, the GBIA values were 4 mg or more. The size of each group of samples is small and the per cent of "false negatives" might well be different with larger numbers, but there are "false negatives" with the GBIA test, especially between 4 and 7 mg per 100 ml. Therefore it becomes critically important to know how many patients with proved PKU who were studied at three days of age or less have had phenylalanine values under 7 mg per 100 ml. Using data compiled by Guthrie[6] and by Hsia and coworkers,[8] two of 24 proved PKU patients first tested at three days of age or less had less than 7 mg of phenylalanine per 100 ml. Extrapolating from our admittedly small series, there would be a 45 per cent chance of failing to detect them at this age by the GBIA method, a grave risk indeed when the harvest is one in ten thousand tests.

All data available to us indicate that persons with PKU attain blood phenylalanine levels well above 7 mg per 100 ml not long after birth—but the question is, how long? If, in using the GBIA method, 4 mg per 100 ml is employed as the level of suspicion, single tests at one week of age, or a second follow-up test within four weeks of age would probably eliminate the risk of "false negatives," based on known data.

Effects of Diet

Another possible solution involves utilization of the effects of diet. In the course of our study of premature infants, we acquired data on 44 who were given an Olac formula and 42 fed Similac. Table 4 shows that the infants who were fed Olac had a mean blood phenylalanine level of 4.53 mg per 100 ml, while for the Similac-fed infants it was 2.21 mg, the difference being highly significant. The data on the Olac-fed infants also showed that 17 were given a supplement of vitamin C, and 27 were not. The mean of blood phenylalanine levels for the 17 was 3.71 mg per 100 ml, and for the 27 it was 5.07 mg—again a significant difference. As a form of phenylalanine loading test, the first feeding of newborn infants with formula might be Olac without vitamin C for a period of 24 hours. It is recognized that feeding Olac without vitamin C supplement might lead to more "false positives," although this remains to be determined by trial on mature newborn infants.

Conclusions

Guthrie's bacterial inhibition assay test for blood phenylalanine, profoundly important to the introduction of mass screening for phenylketonuria, is a simple, cheap procedure admirably suited to private local use. Other screening methods are well suited to centralized private or public large volume studies. At the present stage of our investigations

TABLE 4.—*Effect of Diet on Blood Phenylalanine Level of Premature Infants*

Dietary Group	*Number*	*Blood Phenylalanine mean, mg per 100 ml±S.D.*	*Groups Compared*	*Diff. ± S.E. Diff.*	*S.R.*
A. Similac	42	2.21±0.41	.....	.....	....
B. Olac, all	44	4.53±1.79	A & B	2.32±0.28	8.4
C. Olac without vitamin C suppl.	27	5.07±1.87	A & C	2.86±0.36	7.9
D. Olac with vitamin C suppl.	17	3.71±1.11	A & D	1.50±0.75	2.0
			C & D	1.36±0.45	3.0

S.E. diff.=Standard error of the difference; S.R.=Significance ratio. It is common practice to attribute significance to differences with an S.R. of 3 or more.

they appear to be superior to the Guthrie method.

An inexpensive densitometer that was used to compare growth around standard disks on regular and tetrazolium media permits quality control. Possible slight advantages of the tetrazolium medium include smaller coefficients of variation, greater readings and fewer standards discrepancies, all at higher concentrations of phenylalanine only. Under present conditions there is a small but definite risk of "false negative" results in mass screening of infants three days of age or less by the GBIA method. Premature infants fed Olac formula without vitamin C supplement showed a considerable and significant elevation of phenylalanine as compared with those receiving the formula with vitamin C.

Pending improvement of the GBIA test, the risk of "false negative" results can be eliminated by early feeding with a high protein formula such as Olac minus vitamin C supplement, or performing a single test at an age greater than three days, or performing a second follow-up test within one month of birth.

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